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CO-INOCULATION WITH *RHIZOBIUM* AND *BACILLUS* SP TO IMPROVE THE PHOSPHORUS AVAILABILITY AND YIELD OF WHEAT (*Triticum aestivum* L.).

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ABSTRACT

Intensive cropping has resulted-in wide spread deficiency of nutrients in most of the soils and situation is becoming more serious because of a increase in the use of high priced chemical fertilizers and their negative influence on the environment. Exploitation of biological intervention mainly phosphate solublizing bacteria (PSB) has attracted great attention, as they have enormous potential in providing soil phosphorus for plant growth, by increasing the availability of accumulated phosphate through solubilization. A field experiment was conducted to investigate the effect of *Rhizobium* and *Bacillus*, alone and in combination on the yield parameters of wheat. Uniform dose of N and K (160 and 60 kg ha^{-1}), while two levels of P (57 and 114 kg ha^{-1}) were applied as Urea, SOP and SSP, respectively. *Bacillus* and *Rhizobium* were applied as seed coating to wheat (Var. Sehar 2006).Results revealed that number of tillers (370.3 m^{-2}), spike length (13.50 cm), number of grains (46 $spike^{-1}$), grain yield (6171 kg ha^{-1}), biomass (17.00 t ha^{-1}), grain protein (11.84%) and 1000 grain weight (62 g) were higher in co-inoculation of *Rhizobium* and *Bacillus*. It was also recorded that co-inoculation of *Rhizobium* and *Bacillus* improved the grain yield up to 17.5% as compared to control. In single inoculation *Bacillus* gave better result and showed an increase of 7.7% in grain yield. Phosphorus uptake by grains (25.29 kg ha^{-1}) was maximized by co-inoculation followed by *Bacillus* inoculation. Available phosphorus in post harvest sample of soil was recorded (16.27 mg kg^{-1}) which was significantly higher than all other treatments. Results clearly demonstrated that co-inoculation of *Rhizobium* and *Bacillus* sp enhanced the availability of phosphorus and exert positive effect on the growth and yield of crop.

Key words: Available phosphorus, *Bacillus,* Co-inoculation*, Rhizobium*, Wheat.

INTRODUCTION

Phosphorus is generally deficient in most soils (Batjes, 1997) because almost 75–90% of added P-fertilizer is precipitated by metal cation complexes in calcareous soils of Pakistan (Stevenson, 1986; Hinsinger, 2001). Further, it has also been speculated that the amount of total phosphorus has been increased to such an extent in arable soils which is sufficient to sustain utmost crop yields worldwide for about 100 years (Goldstein *et al.,* 1993).Use of microbial inoculants, overcome the ecological problems, enhance the nutrient availability and nutrient use efficiency (Torsvik and Ovreas, 2002).

Phosphate solubilizing bacteria are used as biofertilizers since long (Krasilinikov, 1957). Species of the genus *Bacillus*, *Pseudomonas, Aspergillus* and *Penicillium* have been identified by many workers as P-solubilizers (Seshadri *et al.,* 2004; Wakelin *et al.,* 2004). These microbes secrete different types of organic acids like carboxylic acid (Deubel and Merbach, 2005) lowering the pH in the rhizosphere (Fankem *et al.*, 2006), consequently mineralize / solubilize the organic / inorganic phosphorus (Pradhan and Sukla 2005; Chen *et al.*, 2006) and hence increased the crop yields (Gull *et al.*, 2004). Microbial inoculants assimilated the soluble phosphorus in plants and prevent it from adsorption or fixation (Khan and Joergensen 2009). This bioavailability of soil inorganic phosphorus in the rhizosphere varies considerably with plant species and nutritional status of soil (Hoflich *et al.,* 1995).

Rhizobium is an important symbiont for legumes but it plays am important role with nonlegumes by producing growth hormones. The first important step for producing growth hormone is root colonization of beneficial bacteria with plants (Kloepper and Beauchamp, 1992). Rhizobia and bradyrhizobia are able to colonize and survive in the rhizosphere of the non legumes plant to act as PGPR in the rhizosphere of non-host legumes and non-legumes (Wiehe and Höflich, 1995). In the cereal-legumes crop rotation, inoculation of the preceding cereal crop with *Rhizobium* sp significantly increased the following legume crop (Gaur *et al.*, 1980). Solubilization of inorganic phosphate is carried out by a large number of strains of *Rhizobium* and *Bradyrhizobium* (Halder and Chakrabartty, 1993). Phosphorus from organic compounds is released by *Rhizobium leguminosarum,* through the action of acid and alkaline phosphatase (Abd-Alla, 1994).

Bacillus sp promoted plant growth by a number of mechanisms, including the solubilization of phosphorus and production of phytohormones such as Indole Acetic Acid (Choudhary and Johri, 2009; Lal and Tabacchioni, 2009). *Bacillus* is abundantly found genus

in the rhizosphere and play vital as phosphate solubilizer and growth promoting rhozobacteria (Probanza *et al.*, 2002 and Gutiérrez *et al.,* 2003).

Co-inoculation of PSB with *Rhizobium* stimulated the plant growth more than their alone inoculation depending upon the soil conditions (Perveen *et al.,* 2002; Zaidi *et al.,* 2003). This situation has certainly brought the subject of phosphate solubilization to the front line and dependence on costly mineral fertilizers is going to be lessened in future. Present study was designed to evaluate the effect of single and dual inoculation of N-fixing and P-solubilizing bacteria on the phosphorus availability and yield parameters of wheat and explore the potential of *Rhizobium* as a PGPR for this important non- legume.

MATERIALS AND METHODS

***Isolation of* Rhizobium *and* Bacillus:** *Rhizobium* was isolated from nodules of chickpea, mung, vegetablepea and barseem (Russell *et al.,* 1982). Pink, healthy and undamaged nodules were selected. Immersed nodules in 95% ethanol for 1-4 minutes. Rinsed in sterile water and then by acidified mercuric chloride Solution (0.1% W/V). Washed nodules for 5-6 times in sterile water. Crushed these nodules with sterile forceps under larger drop of sterilized water in a Petri dish. Then immediately transfer the juice of these crushed nodules to the Congored Yeast Manitole Agar (CYMA) media for growth (Vincent, 1970). The rhizobial growth that did not attain the color of Congo red were picked and re-streaked steadily to obtain pure cultures. The purified rhizobial cultures were stored at 4 ± 2 ^{o}C on slants and maintained for further experimentation. In laboratory study wheat seed was inoculated by *Rhizobium,* isolated from above mentioned legumes and placed in the Petri dishes for germination under controlled conditions. Germination assay of wheat showed that *Mezorhizobium ciceri* was better than other and was selected to use in the experiment.

Bacillus was isolated by dilution plate technique from the rhizosphere soil of wheat growing at the Wheat Research Institute, AARI, Faisalabad. For the isolation of *Bacillus,* rhizosphere soil suspension was placed in the oven for heat shock at 80^{o}C for 10 minutes and on cooling, inoculated on the selective medium (Nautiyal, 1999). Plates were incubated at 28 ± 2 ^{o}C for seven days. The growth of *Bacillus* was purified and screened out on the Pikovskaya medium (El-Komy, 2005). From each plate, the growth was picked and sub-cultured repeatedly to get a pure culture. Grams test (Davies *et al*., 1983) and spore formation (Knaysi, 1935) was positive for this pure culture. Then respiration test was conducted through oil film (Claus and Berkeley, 1986) which came negative indicating the possibility of it being *Bacillus megaterium.* The starch hydrolysis test (Vera *et al.,* 1980) and Voges-Proskauer tests were carried out which was positive and negative respectively, confirming it as *Bacillus megaterium* (Ljutov, 1963.) Following standard methods as outlined in Bergey's Manual of Systematic Bacteriology (Krieg and Holt 1984) the pure culture was predicted as *Bacillus megaterium*.

Auxin Biosynthesis and Phosphate solubilization of isolates: Screening of *Mezorhizobium ciceri* and *Bacillus megaterium* (five of each) were carried out for their auxin biosynthesis potential. The isolates of *Mezorhizobium ciceri* were inoculated on the Yeast Mannitol broth and *Bacillus* on Pikovskaya's broth culture for 72 hours. The auxin biosynthesis potential was determined as Indole-3-acetic acid (IAA) equivalents using Salkowski's reagent (2 mL of 0.5M $FeCl_3$ +98 mL of 35% $HClO_4$) as described by (Sarwar *et al.*1992). *Mesorhizobium ciceri* and *Bacillus* isolates, exhibiting the highest auxin biosynthesis were selected for the study of phosphate solubilization.

The solubilization index of *Bacillus* and *Rhizobium* isolates (5 of each) were checked on the Pikovskaya's medium (Pikovskaya, 1948). Out of five, two isolates of *Rhizobium* and three isolates of *Bacillus* were proficient to solubilize insoluble phosphates in the Pikovskaya's medium by forming the halos. The growth and solubilization diameter were determined after incubation at 28 ± 2 ^{o}C for seven days. On the bases of diameter of clearing halo zones, solubilization index (SI) (Vazquez *et al.* 2000) was calculated using the following formulae.

$$SI = \frac{\text{Colony diameter + halozone diameter}}{\text{Colony diameter}}$$

Auxin biosynthesis potential of *Mesorhizobium* ranged from 13.9-20.8 µg g^{-1} whereas *Bacillus* isolates from 2.1-3.0 µg g^{-1}. Isolates of *Rhizobium* and *Bacillus* with highest auxin biosynthesis potential and phosphate solubilization were selected for experiment (Table 1)

Inoculum of *Rhizobium* was prepared in Yeast Mannitol Broth (YMB) medium and *Bacillus* in Pikovskaya medium (Pikovskaya 1948). Both the media were incubated at 28 ± 2 ^{o}C under shaking at 100 rpm for three days. Leaf mold as carrier was processed and sterilized at 121 ^{o}C and 15 psi pressure for one hour and inoculated with broth cultures of *M. ciceri* and *B. megaterium* (10 mL per 100 g of peat) and incubated at 28 ± 2 ^{o}C. It carries 10^8 CFU $g^{-1}$of leaf mold.

Field Experiment: Field study was conducted with medium textured soil having pH 8.0, EC 1.9 dSm^{-1}, nitrogen 0.029% and available phosphorus 9.0 mg kg^{-1} at Soil Chemistry Section, Ayub Agricultural Research Institute Faisalabad. Two P levels viz. 57 and 114 kg ha^{-1} and uniform dose of N and K 160 and 60 kg ha^{-1} were applied. The experiment was laid out in Randomized Complete Block Design (RCBD) with three replications.

Data regarding grain yield, biomass, number of tillers, plant height, spike length, number of grains, 1000 grain weight, nitrogen and phosphorus uptake of grain protein, soil nitrogen and available phosphorus were recorded after harvesting the crop. Nitrogen was determined according to Kjeldhal method (Bremner and Mulvany 1982) while phosphorus by modified Olsen method (Olsen and Sommers 1982). Data were subjected to statistical analysis by following RCBD using standard procedures (Steel *et al.* 1997). The difference among the treatment means were compared by applying the Duncan's multiple range tests (Duncan 1955).

RESULTS

Co-inoculation Effect on Yield Components: Co-inoculation of *Mezorhizobium* and *Bacillus* sp significantly affected the plant growth. Co-inoculation at half dose of fertilizer significantly enhanced the number of tillers(370 m^{-2}) compared to all other treatments while *Bacillus* sp produced 352 tiller m^{-2} and *Rhizobium* sp. 303 at same dose of fertilizer. Co-inoculation showed 40.4% increase in number of tillers m^{-2} compared to control. Variable but non-significant plant height and spike length was observed (Table 2). Plant height was highest with *Mezorhizobium* inoculation (105.6 cm) at full dose of fertilizer while it was 101.5 and 101.6 cm by co-inoculation and *Bacillus* treatment, respectively than control (100.2 cm).

Co-inoculation exhibited maximum spike length i.e. 13.5 cm at half dose of fertilizer while *Bacillus* sp i.e.13.33 cm and *Rhizobium* inoculation 12.54 cm by at full dose of fertilizer. Co-inoculation produced significantly higher number of grains $spike^{-1}$ i.e. 46 than all other treatment and increase in grains $spike^{-1}$ was 28.9% by co-inoculation over control.

Maximum grain yield was observed by co-inoculation than all other treatments. Non-significant increase in biomass yield, grain protein, and 1000 grain weight was observed (Table 3). Co-inoculation produced significantly higher grain yield i.e. 6171 kg ha^{-1} at half dose of fertilizer followed by *Bacillus* i.e. 5964 kg ha^{-1} and by *Rhizobium* i.e. 5933 kg ha^{-1} at full dose of fertilizer. Increase in grain yield by co-inoculation was 17.5% over control.

Co-inoculation produced significantly higher biomass yield (17 tonne ha^{-1}) at full dose of fertilizer followed by *Bacillus* inoculation (16.17 tonne ha^{-1}) and *Rhizobium* (16.0 tonne ha^{-1}) at same dose of fertilizer. Highest grain protein was observed with co-inoculation (11.84%) at full dose of fertilizer followed by *Rhizobium* (11.18%). Increase in grain protein was 13.7% by co-inoculation over control. With regards to 1000 grain weight, *Bacillus* at full dose and co-inoculation at half dose of fertilizer were at par i.e. 62 g followed by *Rhizobium* i.e. 54 g at full dose of fertilizer.

Co-inoculation Effect on Grain and Soil Composition**:** Data about NP uptake by grains is presented in Table 4. Non significant but highest P-uptake was observed with co-inoculation (25.29 kg ha^{-1}) at half dose of fertilizer followed by *Bacillus* inoculation (23.06 kg ha^{-1}) at full dose of fertilizer.

N-uptake by wheat grains was significantly highest (106.80 and114.6 kg ha^{-1}) at fertilizer levels (160-114-60) with *Rhizobium* and co-inoculation respectively which were statistically at par with each other (Table 4) followed by *Bacillus* (105.13 kg ha^{-1}).Increase in N-uptake by co-inoculation was 25.56% over control.

Inoculations of *Rhizobium* and *Bacillus* alone and in combination produced higher soil N and available P as compared to control. The highest but non significant soil N was observed in case of rhizobial and co-inoculation (0.035%) at half dose of fertilizer. Co-inoculation exhibited maximum available P (16.27 mg kg^{-1}) that differed significantly from *Bacillus* and *Rhizobium* inoculation (14.27 and 13.61 mg kg^{-1}respectivly) at half level of fertilizer (Tab.4).Co-inoculation showed 56.1% increase in available P compared to uninoculated control followed by *Bacillus* inoculation (36.9%).

Table 1. Some important features of isolates tested during the investigation.

Isolates	IAA equivalents (µg mL^{-1})	Gram reaction	Solublization Index (SI)
Rhizobium (Chickpea)	20.8	-ve	2.5
Rhizobium (Mung)	17.3	-ve	2.0
Rhizobium (Vegetable pea)	19.0	-ve	2.1
Rhizobium (Barseem)	18.0	-ve	2.0
Bacillus megaterium	3.1	+ve	3.7

Table 2. Effect of *M. ciceri* and *B.megaterium* on physical parameter of wheat

Average of 3 repeats

Treatments	Number of tillers(m^{-2})		Plant height (cm)	
	57 kg P ha^{-1}	**114 kg P ha^{-1}**	**57 kg P ha^{-1}**	**114 kg P ha^{-1}**
Control	263.6 d	305.7 c	97.8	100.2
M.ciceri inoculation	303.0 c	326.3 c	101.7	105.6
B.megaterium inoculation	352.3 ab	319.3 bc	99.2	101.6
M.ciceri+B.megaterium	370.3 a	313.0 c	101.2	101.5
LSD	38.39		NS	
	Spike length(cm)		Number of grains($spike^{-1}$)	
Control	10.45	11.33	35.7 d	37.3 d
M.ciceri inoculation	12.42	12.54	41.3 bc	40.3 c
B.megaterium inoculation	13.12	13.33	42.3 bc	42.7 bc
M.ciceri+B.megaterium	13.50	13.20	46.0 a	43.7 ab
LSD	NS		2.52	

†*rhizobium + bacillus* inoculation in (1:1)*means sharing similar letter(s) in a column do not differ significantly at $p<0.05$ according to Duncan's multiple range test

Table 3. Effect of *M. ciceri* and *B.megaterium* on biomass, grain yield and 1000 grain weight and grain protein of wheat

Average of 3 repeats

Treatments	**Grain yield (kg ha^{-1})**		**Biomass (tonne ha^{-1})**	
Control	57 kg P ha^{-1}	114 kg P ha^{-1}	57 kg P ha^{-1}	114 kg P ha^{-1}
	5250.7 d	544.3 cd	14.60	15.16
M.ciceri inoculation	5601.7 c	5933.7 ab	15.67	16.00
B.megaterium inoculation	5656.7 c	5964.0 ab	15.87	16.17
M.ciceri+B.megaterium	6171.0 a	6010.0 ab	16.47	17.00
LSD	214.9		NS	
	Grain protein (%)		1000 grain weight (g)	
Control	10.37	10.41	50	53
M.ciceri inoculation	11.02	11.18	53	54
B.megaterium inoculation	10.60	10.95	53	62
M.ciceri+B.megaterium	11.69	11.84	62	55
LSD	NS		NS	

†*rhizobium + bacillus* inoculation in (1:1)

*means sharing similar letter(s) in a column do not differ significantly at $p<0.05$ according to Duncan's multiple range tests

Table 4. Effect of *M. ciceri* and *B.megaterium* on grain and soil composition

Average of 3 repeats

Treatments	**Grain P-uptake (kg ha^{-1})**		**Grain N-uptake (kg ha^{-1})**	
	57 kg P ha^{-1}	**114 kg P ha^{-1}**	**57 kg P ha^{-1}**	**114 kg P ha^{-1}**
Control	14.7	16.71	87.67 d	91.27 c
M.ciceri inoculation	18.06	20.37	99.37 c	106.80 a
B.megaterium inoculation	21.24	23.06	96.50 d	105.13 b
M.ciceri+B.megaterium	25.29	25.24	116.20 b	114.60 a
LSD	NS		4.286	
	Soil N (%)		Available P (mg kg^{-1})	
Control	0.0.31	0.033	10.42 e	10.96 c
M.ciceri inoculation	0.035	0.034	13.61 cd	12.80 d
B.megaterium inoculation	0.031	0.033	14.27 bc	12.96 d
M.ciceri+B.megaterium	0.035	0.034	16.27 a	14.83 b
LSD	NS		0.9937	

†*rhizobium + bacillus* inoculation in (1:1)

*means sharing similar letter(s) in a column do not differ significantly at $p<0.05$ according to Duncan's multiple range test

DISCUSSION

Rhizobium leguminosarum was isolated from the nodules of different legumes by method reported by Vincent (1970), while *Bacillus* sp. was isolated by preparing the serial dilutions from the rhizosphere of wheat by procedure formulated by Nautiyal, (1999). Their efficiency was checked in the laboratory by gram test, measurement of IAA equivalents and phosphate solubilization on Pikovskaya's medium (El-Komy, 2005) (Table 1). Previous studies revealed the auxin production and plant growth promotion by microbial population (Gull *et al.,* 2004; Martins *et al.,* 2004).

In present study co-inoculation of *Rhizobium and Bacillus* and two levels of phosphorus were applied to the wheat. Results showed significant increase in number of tillers plant^{-1} by co-inoculation of *Rhizobium* and *Bacillus* at half dose of fertilizer (Table 2). It is because of the fact that microbial growth and activity become slow at high level of nutrients and work efficiently under moderate to low fertile condition. It is supported by the previous finding that growth promotion of maize and lettuce in moderately fertile soils is due to phosphate solubilization by strains of *Rhizobium leguminosarum* (Chabot *et al.*, 1996a). Numbers of tillers are directly related to the yield.

Non-significant increase in plant height and spike length was observed. Excess nitrogen may cause increase in plant height, succulence and lodging. Phosphorus gives strength and maturity to the crop. In our experiment phosphorus was not only supplied as fertilizer but also solubilized by the microbes. Previous work supported our finding that plant height increased by microbial inoculation Sharma *et al.* (2007) and significant increase in spike length was observed by Afzal and Bano (2008).

The ultimate yield of wheat is the grain. So the healthy grains per spike are of great importance. Number of grains spike^{-1}increased by co-inoculation of *Rhizobium* and *Bacillus* (Table 2) due to availability of sufficient nutrition other than that provided by fertilizer. It is the well established fact that *Rhizobium* and *Bacillus* sp synthesize growth hormones which have positive effect on plant growth. Plant hormones producing microbes enhance the root surface area and thus resultes in more nutrient uptake. (Yuming *et al,*. 2003).

The single inoculation of *Rhizobium* as well as *Bacillus* showed statistically significant result compare to control because they are not only phosphate solubilizer but also produce growth hormones. In co-inoculation they perform even better without suppressing the effect of each other. Results are supported by the previous study that dual inoculation increased in wheat yield (Galal, 2003). Literature as well as practical studies suggests that co-inoculation shows better results whenever there is a synergistic relationship between the microbes. In the present study the microbial inoculation of both microbes aims at increasing phosphorus solubilization in the root zone. As both the microbes are working towards the same aspire, result would be positive. So co- inoculation is better than separated application.

Results regarding Biomass, grain protein and 1000 grain weight are given in (Table, 4). Biomass yield may increase due to excessive vegetative growth which may delay the maturity. In present study there is non significant increase in biomass grain protein and 1000 grain weight. In co-inoculation seed was healthy and weigh more than control and single inoculation as well. Similar findings were given previously that co-inoculation increased the 1000 grain weight than *Rhizobium* alone Askary *et al.* (2009).

Nutrient uptake by grain depends on availability of nutrient. *Rhizobium* and *Bacillus* are the most important phosphate solublizers. Co-inoculation of these microbes helped plant to take phosphorus 25.29 kg ha^{-1} compare to control (14.7 kg ha^{-1}).The increase in phosphorus uptake is 72% by co-inoculation over control.

Nitrogen uptake by grain is positively affected by inoculation. Results are supported by previous finding that biofertilizers with half dose of NP fertilizer give the crop yield as with full doze of NP fertilizer Jilani *et al.* (2007). Many researchers reported increased seed P content by phosphate solubilizing microorganisms Son *et al.* (2006) and Zaidi *et al.* 2004).According to Kumar and Chandra (2008) phosphate solubilizing bacteria significantly increased the uptake of both nitrogen and phosphorus by grain and straw of lentil.

In our study variable but non significant soil nitrogen contents were observed by inoculation (Tab 4). Available phosphorus was significantly affected by inoculation. Microbes such as *Bacillus* and *Rhizobium*, produce organic acids thus lower the soil pH, solubilize the precipitated phosphorus and make it available to plant. Similar results were given by Khan *et al.* (2006) they reported that dual inoculation of *Rhizobium* and *Bacillus* outcome more soil nitrogen and the available phosphorus.It is concluded that bioavailability of precipitated phosphorus is possible by bacteria such as *Bacillus* and *Rhizobium.* Co-inoculation of N-fixing and P-solubilizing bacteria positively effects the growth and yield of wheat by providing growth hormone and increasing the N and P uptake by plant. Co-inoculation also improves the nutrient status of soil thus provide healthy environment for the next crop.

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